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**Profiling inflammatory response in lesions of cutaneous leishmaniasis patients using a non-invasive sampling method combined with a high-throughput protein detection assay**

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## Highlights

- A non-invasive sampling method combined with a multiplexed protein assay was developed for evaluation of cytokine and chemokines in the lesions of cutaneous leishmaniasis patients.
- Various inflammatory cytokines, chemokines and other proteins were detected in the lesions of cutaneous leishmaniasis patients.
- This non-invasive sampling method combined with a multiplexed protein assay has implications for evaluation of skin inflammatory responses in cutaneous leishmaniasis and other skin diseases.

## **Abstract:**

### **Background:**

Cutaneous leishmaniasis (CL) is an infection caused by *Leishmania* (*L.*) protozoa transmitted through the bite of infected sand fly. Previously, invasive sampling of blood and skin along with low throughput methods were used for determination of inflammatory response in CL patients.

### **Aims/Methodology**

We established a novel approach based on a non-invasive adhesive tape-disc sampling combined with a powerful multiplexing technique called proximity extension assay for profiling 92 inflammatory cytokines, chemokines and surface molecules in the lesions of CL patients infected with *L. tropica*. Sample collection was done non-invasively by using adhesive tape-discs from lesion and normal skin of 33 *L. tropica* positive patients.

### **Results**

Out of 92 inflammatory proteins, the level of 34 proteins was significantly increased in the lesions of CL patients compared to their normal skin. This includes the chemokines CCL2, CCL3, CCL4, CXCL9, CXCL10, CXCL11 and CXCL5, together with the interleukins IL-18, IL-8, IL-6, LIF and OSM. The remaining significantly changed inflammatory proteins include 7 surface molecules and receptors: CD40, CDCP1, 4E-BP1, TNFRSF9, CD5, IL-18R1 and OPG as well as 16 other cytokines and proteins: MMP-1, CSF-1, VEGFA, uPA, EN-RAGE, LAP TGF- $\beta$ 1, HGF, MMP-10, CASP-8, TNFSF14, STAMPB, ADA, TRAIL and ST1A1. Further, 13 proteins showed an increasing trend, albeit not statistically significant, in the CL lesions, including TGF- $\alpha$ , CCL23, MCP-2, IL-12B, CXCL6, IL-24, FGF-19, TNF $\beta$ , CD6, TRANCE, IL10, SIR2 and CCL20.

### **Conclusion**

We herein report a novel approach based on a non-invasive sampling method combined with the high-throughput protein assay for profiling inflammatory proteins in CL lesions. Using this approach, we could profile inflammatory proteins in the lesions from CL patients. This new non-invasive approach may have implications for studying skin inflammatory mediators in CL and other skin disorders.

## **Keyword**

Cutaneous leishmaniasis, non-invasive sampling, Proximity extension assay, Inflammation biomarkers

## **Abbreviation**

ADA: Adenosine deaminase

ARTN: Artemin

Beta-NGF: Nerve growth factor

CSF-1: Colony stimulating factor 1

CST5: Cystatin D

DNER: Delta/Notch Like EGF Repeat Containing

4E-BP1: Eukaryotic initiation factor 4E-binding protein 1

EN-RAGE LAP (TGF-beta-1): Advanced glycation end-products binding protein

FGF: Fibroblast growth factor

GDNF: Glial cell line-derived neurotrophic factor

HGF: Hepatocyte growth factor

LIF: Leukemia inhibitory factor; IL-6 family

MCP-1: Monocyte chemoattractant protein-1

MMP: Matrix metalloproteinase

NRTN: Neurturin

OPG: Osteoprotegerin, member of TNF family

OSM: Oncostatin M, IL-6 family

PDL-1: Program death legend 1

98 ST1A1: Sulfotransferase1A1  
99 STAMPB: STAM binding protein  
100 SIR2: SIR 2 like protein 2  
101 TNFRSF: Tumor necrosis factor receptor superfamily memberTGF- $\beta$ : Transforming growth  
102 factor-beta  
103 TRAIL: TNFSF10  
104 TRANCE: TNF-related activation-induced cytokine  
105 uPA: Urokinase-type plasminogen activator  
106 VEGFA: Vascular Endothelial Growth Factor A  
107

## 1. Introduction

Cutaneous leishmaniasis (CL) is a skin infection caused by *Leishmania* protozoa transmitted by the bite of an infected sand fly. More than twenty different *Leishmania* species can cause the disease [1]. As a multifaceted disease, CL has different clinical manifestations ranging from self-healing papules to permanent non-healing lesions. Although CL is not a fatal disease, it causes serious socio-economic burden in endemic areas. CL infections are mostly zoonotic with the exception of the infection caused by *L. tropica*, which is often anthroponotic. Following the sand fly bite, the outcome of the disease is dependent on the extent to which the parasite propagates, the host immune response, and the sand fly characteristics. Studying the site of infection is pivotal in understanding the underlying mechanism of the disease and the host immunity. Due to restrictions associated with collection of skin biopsies from humans, including ethical limitations and the risk of secondary infection, the majority of the previous studies were centered on the use of mouse models of the disease [2-8]. However, some studies have limitedly investigated the immune responses in the skin lesions of CL patients [8-13]. There is currently limited information available on the immunological responses in the skin lesions of CL patients.

Upon injection of the metacyclic form of the parasite into the skin, various inflammatory cells such as macrophages, monocytes, neutrophils and dendritic cells are considered to play roles in immunity to infection and tissue damage [14]. *Leishmania* parasites are rapidly engulfed by infiltrating neutrophils that are deemed to act as the first target cells. Macrophages are among the first inflammatory cells that encounter the parasites, which may kill the parasites by production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [15]. Dendritic cells were also shown to play a key role in the immunity to the parasite [16]. It is clear that the balance between Th1 and Th2 responses is critical in the control of the disease. A Th1 response is

considered to contribute to clearing the infection, whereas a Th2 dominated response is deemed to be associated with non-healing cases [17, 18]. We have recently developed a new non-invasive tape-disc sampling method for the diagnostics of CL [19, 20]. Building on this approach, we herein established the potential of this tape-disc sampling method combined with a high throughput multiplexing protein detection assay (Proximity Extension Assay technology) [21, 22] for studying the host inflammatory response to *L. tropica* infection in human skin lesions. This novel non-invasive approach has implications for studying local immune responses in skin diseases. A schematic representation of the approach employed in the present work is summarized in Figure 1.

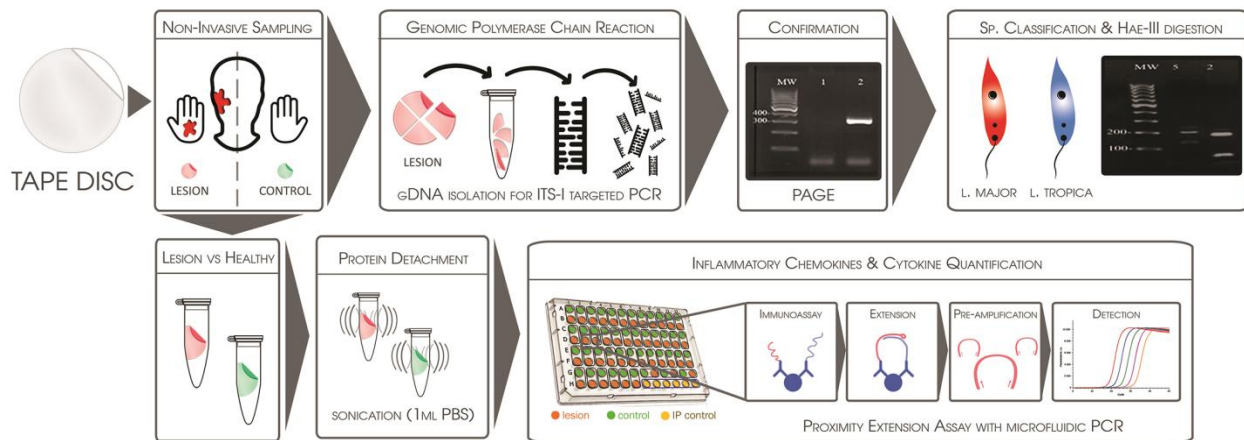


Fig. 1: A schematic representation of tape-disc sampling method combined with Proximity Extension Assay and ELISA for assessment of inflammatory mediators and specific antibody in the lesions of CL patients, respectively.



## 2. Materials and Methods

### 2.1. Ethics

This study was authorized by the Ethics committee of the Pasteur Institute of Iran 27/01/2019, TP-9566. A written informed consent form was signed by the patients or their guardians (for those with 18 years of age) before sampling.

### 2.2. Study design, sample collection and diagnostics

A cross-sectional study enrolling 33 CL patients (25 acute and 8 chronic cases) with total of 66 specimens (including lesions and their healthy skin counterparts) was conducted. The acute cases were determined as those with active CL lesions for less than 12 months, while chronic cases were those with active CL lesions for one year or more. Acute cases with no treatment and treated chronic cases were selected for this study. Sampling was done in Abobargh health care center for CL, North Khorasan province, Mashhad. This area is highly endemic for *L. tropica*, and patients are referred to this center for diagnosis and receiving their free medication and follow-up of their treatment. Sample collection from lesions and normal skins was done non-invasively by using 22mm adhesive tape-discs (D-squame, CuDerm Corporation, Texas, USA) as described previously [19]. Samples were stored at -20°C until further use. Genomic DNA from the tape-discs was extracted using DNeasy Qiagen Blood and tissue kit (Qiagen, Germany), and subsequently subjected to *L. tropica* specific PCR-RFLP as previously described [19].

### 2.3. Protein quantification using multiplex inflammation panel

Supernatants retrieved from tape disc samples of CL lesion samples and their healthy skin counterparts were analyzed using the Olink 92-plex Inflammation panel (performed by Olink Bioscience, AB). This panel includes 92 chemokines, cytokines and surface proteins involved in inflammation. Briefly, tape-discs from lesions and healthy skins were sonicated in 1ml of sterile Phosphate Buffer saline (PBS, pH 7.4) for 10 minutes. The supernatant was collected and divided into 100µl aliquots and stored at -20°C until further use. Protein suspensions (25µl) were quantified using PEA and normalized with inter-plate controls and internal analyte controls to generate normalized protein expression values (NPX) in a log<sub>2</sub> scale. PEA is based on a pair of oligonucleotide strands coupled with specific antibodies which pairwise bind to the target biomarker. Polymerase chain reaction (PCR) was carried out by adding DNA polymerase to the PEA mixture [22].

### 2.4. Antigen lysate preparation for ELISA

*L. tropica* (MOHM/IR/Khamesipour-Mashhad) parasites were cultured at 26°C in Medium 199 (M199; Sigma), pH 7.2, supplemented with 10% heat-inactivated FCS (Gibco), 0.1mM adenosine (Sigma), 40 mM HEPES (Sigma), and 50µl/ml gentamicin (Sigma). *Leishmania* parasites were retrieved by centrifugation and washed in PBS (1000 g/10min), and 3x10<sup>8</sup> parasite/ml were used for 10 times freeze-thaw (liquid nitrogen followed by 37°C water bath) cycles. Pierce<sup>TM</sup> BCA Protein Assay Kit (Thermo fisher scientific) was used to measure the total protein concentration of the parasite lysate.

## 2.5. Detection of *L. tropica* specific IgG antibodies

The sonicated tape-disc supernatant was used for detection of *L. tropica* specific IgG antibody [23]. MaxiSorp flat bottom 96-well plate (Sarstedt) was coated with 10µg/ml of *L. tropica* lysate in 100 µl/well and incubated at 4°C overnight. Un-bound leishmanial antigens were washed three times with 300µl/well of 0.05% Tween 20 (Sigma) in PBS. To reduce the non-specific binding, the wells were blocked with 300 µl/well of 1% IgG-free bovine serum albumin (BSA; Sigma) in PBS for one hour at room temperature. The wells were washed three times, and 100 µl of 1:2.5 diluted disc supernatants with dilution buffer (0.05% Tween and 1% BSA in PBS) was added to each well and incubated for one hour at room temperature. The plate was washed and then 100 µl/well of Horseradish Peroxidase (HRP) conjugate Goat-Anti-human IgG Fc antibody (diluted 1:20000 with blocking solution) (Sigma) was added and incubated in dark for 1 hour. The plate was then washed 4 times, and 100 µl/well HRP substrate, TMB (ebioscience) was added. After 15 minutes, 100 µl/well of 1N H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction and the plates were read at 450 nm absorbance (BioTek, ELx800).

## 2.6. Statistical analysis and data visualization

Age distribution of patients was reported as mean  $\pm$  SD. Statistical comparison of differences between lesion and healthy skin was performed using Mann-Whitney U test (Graph Pad Prism version 5.0) for both PEA and ELISA assays.

PEA analyses were performed separately for acute and chronic lesions compared to healthy skin samples using Mann-Whitney U test in R version 3.6.0 (R Core Team; 2019). The PEA analysis results were then visualized in a Circos plot generated using CIRCOS v 0.69-6 [24] to visualize

213 the similarities and differences between inflammation related proteins in the lesional samples and  
214 the normal samples. *P*-values < 0.05 were considered statistically significant.

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216

### 3. Theory/Calculation

Cutaneous leishmaniasis patients suffer from delayed diagnosis, mis-diagnosis and consequently a postponed treatment which may cause severe tissue damage and the formation of hypertrophic scars on the patient's skin for the rest of their lives. It could be due to the lack of knowledge related to the immunological interactions. Nowadays, finding proper biomarkers as an early diagnostic tool is a need for various diseases. Biomarkers are mostly found in blood and body fluids, most likely due to the ease of sample collection, and in the case of CL they need to be investigated in the lesions. In this work we report development of a non-invasive sampling method combined with a high-throughput protein detection assay for profiling proteins involved in the inflammatory response on CL lesions, which may be beneficial for studying inflammatory proteins in other skin diseases alike.

### 4. Results

#### 4.1. Diagnosis determination of CL patients

Twenty five acute patients, including 14 men and 11 women, with mean age of 27.5 (IQR: 7.5, 47) and median lesion duration of 4 months and 8 chronic patients, including 5 men and 3 women, with mean age of 44.5 (IQR: 11, 51.75) and median lesion duration of 29 months were included in the study (Table 1). To differentially diagnose the *Leishmania* species, DNA samples isolated from 33 non-invasively collected tape-discs obtained from acute and chronic patients were subjected to *L. tropica* specific PCR-RFLP. All 33 collected samples were diagnosed and characterized as *L. tropica* (Data not shown).

Table 1: Characteristic profile of *L. tropica* leishmaniasis patients

Type of lesion	Age, median, year (IQR)	Sample size n (% male)	Duration of lesion (median, month)	Lesion location	Number of Lesion	Number of cases
<b>Acute</b>	27.5 (IQR: 7.5, 47.5)	25 (56)	4	Head: 11 Hand: 15 Foot: 2	Single: 14 Multiple: 11	25
<b>Chronic</b>	44.5 (IQR: 11, 51.75)	8 (62)	21	Head:6 Hand:2 Foot:2	Single: 5 Multiple: 3	8

Table 1: Characteristic profile of *L. tropica* infected cutaneous leishmaniasis patients. Values are presented as median (IQR 25- 75), IQR: Interquartile range.

#### 4.2. Profiling inflammatory-related proteins in acute and chronic CL lesions

To investigate the difference in expression of inflammatory proteins between lesions and their normal skin, 92 soluble factors included in Olink® inflammatory panel were selected. The disc supernatants obtained from lesion and normal skin samples were subjected to the PEA inflammatory panel.

Out of the 92 measured proteins, 34 (37%) proteins showed significantly ( $P<0.05$ ; Mann-Whitney U test) higher levels in the acute lesions compared to their normal skin counterparts

(Fig 2 and Fig 3a). These proteins are involved in Apoptotic process, Cell activation involved in immune response, Cell adhesion, Cellular response to cytokine stimulus, Chemotaxis, Extracellular matrix organization, Inflammatory response, MAPK cascade, Regulation of immune response, Response to hypoxia, Secretion, and other biological processes.

Figure 2:

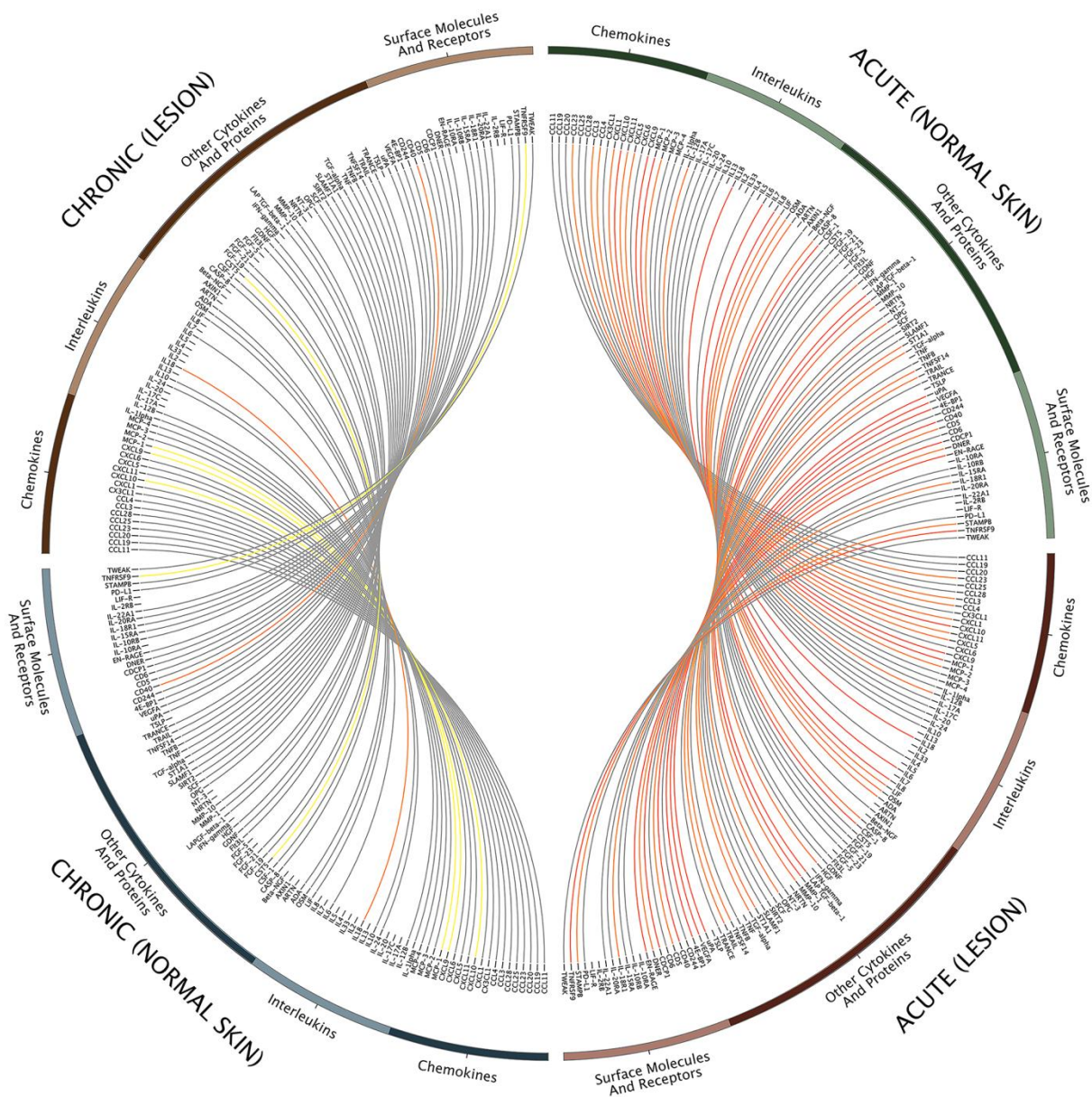
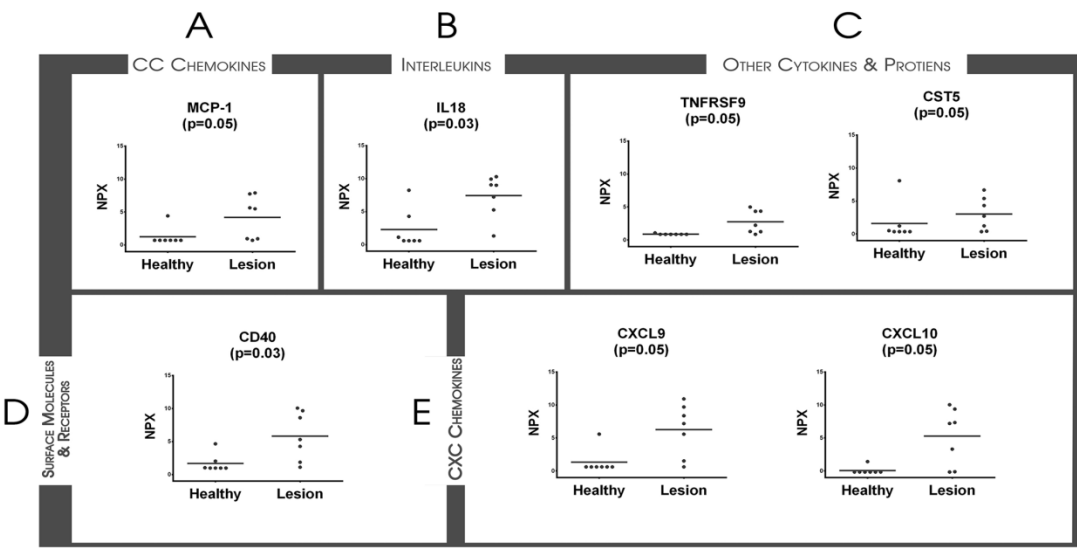




Fig 2: CIRCOS plot depicting similarities and differences in the levels of the inflammation-related proteins in lesions of acute and chronic CL patients compared to normal skin. Red lines:  $P < 0.01$ , orange lines:  $P < 0.05$  and yellow lines:  $P < 0.1$ . Outer circles represent the family group of each protein, including Chemokines, Interleukins, other cytokines and proteins and also Surface Molecules and Receptors.

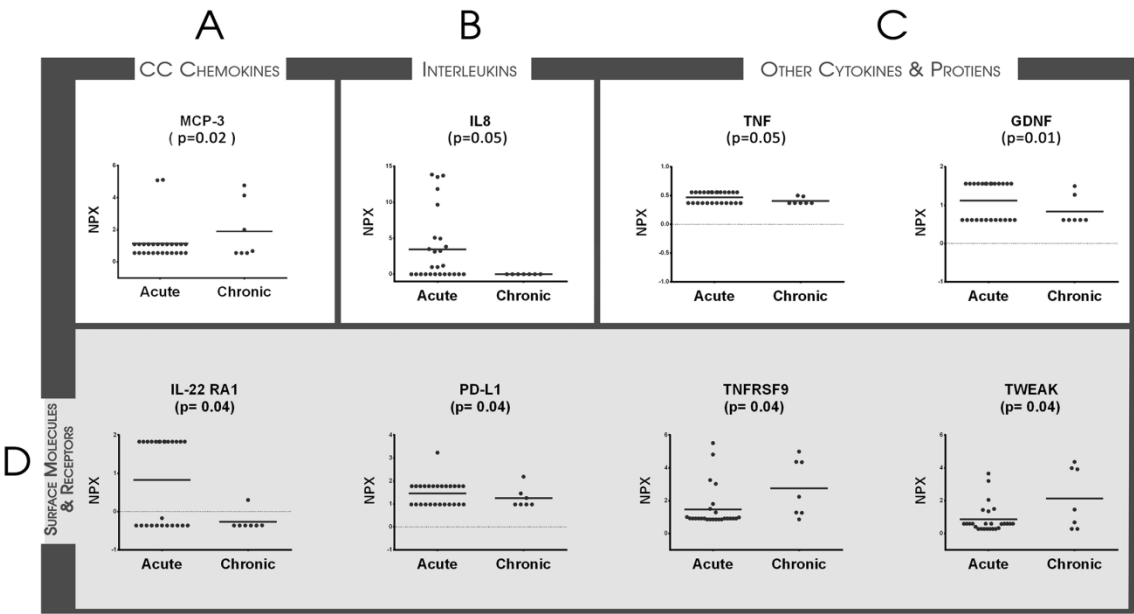


314 Figure 3b:



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316 Figure 3c:



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Figure 3: The inflammation-related proteins significantly changed in the lesions of the CL patients compared to their healthy skin counterparts. Normalized protein Expression Units (NPX) values of healthy skin compared to leishmaniasis lesion are plotted. Proteins are classified into five groups based on their functions. A. CC Chemokines; B. CXC Chemokines; C. Interleukins; D. Surface molecules and receptors; E. other cytokine and proteins. *P*-value of  $P < 0.05$  was considered statistically significant and the exact *p*-value is indicated in each graph.

a: Acute patients' (Lesion, *n*=25) inflammation-related proteins compared to their own healthy skin (Healthy, *n*=25). Fig 3b: Chronic patients' (Lesion, *n*=8) inflammation-related proteins compared to their own healthy skins (Healthy, *n*=8).

Fig 3c: Comparison of inflammation-related proteins in acute lesion-healthy skin (*n*=25) to chronic lesion-healthy skin (*n*=8). Both lesion groups have been corrected by using respective healthy skin.

Among these proteins, eight were chemokines: MCP-1 (CCL2), CCL3, CCL4, CXCL9, CXCL10, CXCL11 and CXCL5, together with five interleukins: IL-18, IL-8, IL-6, LIF and OSM (Fig 2 and Fig 3a).

The other important groups of proteins which showed enhanced levels in the acute lesions include the surface molecules and receptors CD40, CD44, CD5, IL-18R1 and OPG, together with 16 cytokines and other proteins consisting of MMP-1, MMP-10, CSF-1, uPA, EN-RAGE, LAP TGF- $\beta$ 1, HGF, VEGFA, CASP-8, TNFSF14, STAMPB, ADA, TRAIL and ST1A1 (Fig 2, Fig 3a).

Further, 13 proteins showed an increasing trend, albeit not statistically significant: CCL20, CCL23, CXCL6, MCP-2, IL-12B, IL-24, FGF-19, TGF- $\alpha$ , TNF- $\beta$ , CD6, TRANCE, IL-10RB and SIR2. DNER was the only marker with significant decrease in the lesions (Fig 2 and Fig 3a).

In chronic patients, 7 proteins had significantly higher levels of expression in the lesions as compared to their healthy skin counterparts (Fig 2 and Fig 3b). These include CCL2, CXCL9, CXCL10, IL-18, CD40, TNFSF9 and CST5 (Fig 2 and Fig 3b). CST5 was, however, exclusively detected in the chronic lesions compared to healthy skin and acute ones. IL-8, IL-22 RA1, PD-

L1, TNF and GDNF showed significantly increased levels in the acute lesions compared to those of the chronic lesions. MCP-3, TNFRSF9 and TWEAK were decreased significantly in the acute lesions (Fig 3c).

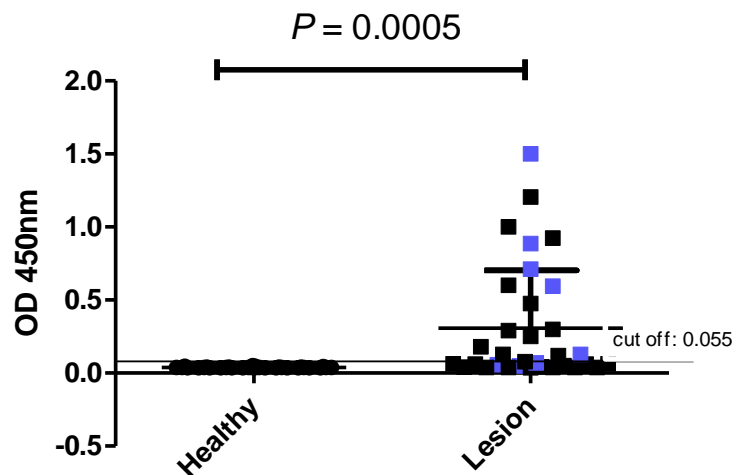
It is worth mentioning that amongst 92 cytokines and chemokines measured, 15 of them including interleukins IL-2, IL-5, IL-7, IL-13 and IL-33, some receptors such as IL-10RA, IL-2RB and ILF-R, cytokines: IFN- $\gamma$ , TSLP and a group of growth factor related proteins such as ARTN, NRTN, Beta-NGF and FGF-5 were found to be undetectable in 99% of the samples in both lesions and their healthy control counterparts.

#### **4.3. Evaluation of *L. tropica* specific IgG antibody in the tape-discs from CL lesions**

Next, we sought to evaluate the potential of the tape-disc sampling method in the retrieval of *L. tropica* specific IgG antibodies from lesions of acute and chronic CL patients. To do this, the supernatants of tape-discs collected from the lesions and their normal skin counterparts were subjected to *L. tropica* specific IgG antibody ELISA. *L. tropica* specific IgG antibody was detected in 17 out of 25 acute patients' samples, and in 6 out of 8 chronic cases (Fig 4). This result shows the potential of the tape-disc sampling for evaluation of *L. tropica* specific IgG antibody responses in CL lesions.

371 Figure 4:

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375 Figure 4: *L. tropica* specific IgG antibody levels in the lesions of acute and chronic CL patients.  
376 Tape-discs' supernatants retrieved from lesions and healthy skins of acute (n=25; colored in  
377 black) and chronic (n=8; colored in blue) cutaneous leishmaniasis patients (n=33). Squares  
378 represent the average mean and SD in duplicate. Cut- off value was adjusted three times higher  
379 than the mean absorbance obtained from normal skins.

380

381

## 5. Discussion

We herein report, for the first time, the establishment of a non-invasive tape-disc sampling method combined with a multiplexed protein detection assay for the assessment of inflammatory mediators in the skin lesions of CL patients. Using this novel approach, we have observed significant differences in the expression of five important groups of inflammatory proteins in the lesions of acute CL patients compared to their normal skin. These include CC chemokines, CXC chemokines, interleukins, surface molecules and immune receptors. This finding is in line with previous reports on the recruitment of monocytes, macrophages, DCs, NK cells and T cells to the site of CL infection [25]. The CXC chemokines CXCL1, CXCL9, CXCL10 and CXCL11 showed significantly higher levels in the acute CL lesions, CXCL1 acts as an activator and recruiting agent of neutrophils into the CL lesion [26]. CXCL9, CXCL10 and CXCL11 have previously been shown to be up-regulated in localized CL caused by *L. braziliensis*. Conversely, these chemokines were reported to be down-regulated in diffuse CL caused by *L. amazonensis* [27]. The CC chemokines CCL2, CCL4 and CCL5 were reported to serve as putative biomarkers of high parasite load in the lesions of *L. infantum* infected dogs [28]. Interleukins whose expression increased significantly in CL lesions include IL-6, IL-8, IL-18, OSM and LIF. The skin level of IL-6 was previously reported to be elevated in CL [11], psoriasis and leprosy [29]. IL-8 has been shown to play a major role in tissue damage in CL [30]. However, the role of the Th1/Th2 regulating cytokine IL-18 in the healing process of CL lesion is controversial [17, 30]. IL-18/IL-18R interaction promotes activation of NK cells, and induces the production of IFN- $\gamma$ , and was shown to contribute to protection against *L. major* [31]. The members of IL-6 family, including OSM, LIF and IL-6 were shown to be involved in skin inflammatory diseases such as psoriasis and atopic dermatitis [32].

405 We could also show an increasing trend in the protein levels of several surface molecules and  
406 receptors such as 4E-BP1, CD-40, TNFRSF9, OPG, CDCP1, CD5, and IL-18R1. 4E-BP1 has  
407 been reported to promote the persistence and survival of the parasite in CL lesions [33]. CD40 as  
408 a member of TNFR superfamily plays an important role in antigen presentation to control CL  
409 [34]. CD5<sup>+</sup> B cells have been shown to increase in the active phase of the disease [35]. The other  
410 cytokines and proteins which showed significantly increased levels in the lesions of the CL  
411 patients were CSF1, VEGFA, uPA, EN-RAGE, LAP TGF- $\beta$ 1, DNER, HGF, MMP-1, MMP-10,  
412 CASP-8, TNFSF14, STAMPB, ADA, TRAIL and ST1A1. The CSF1 and its receptor signaling  
413 have been shown to play a crucial role in development of dermal macrophages [36]. The  
414 expression of VEGFA and its receptor was reported in CL lesions [37]. The inflammatory  
415 mediator TNFSF14 was shown to contribute to the control of cutaneous and visceral  
416 leishmaniasis [38]. The pro-inflammatory antimicrobial EN-RAGE was reported in the brain of  
417 mice infected with *L. amazonensis* [39]. MMP-1 has been reported as one of the most highly up-  
418 regulated proteins in CL [40]. The serine protease uPA was found in tissue extracellular matrix,  
419 and is expressed in the amastigote of *Leishmania* parasites [41]. TRAIL accompanied by FAS-  
420 ligand was found to mediate keratinocyte damage, and was detected in the lesions of *L.*  
421 *aethiopica* infection [42]. An increased ADA level was also reported in the lymphocytes of CL  
422 patients [11].

423 Blockade of CASP-8 has been reported to enhance the immunity against *L. major* infection [43].  
424 The activation of LAP TGF- $\beta$ 1 has been reported in pathology of CL [44]. Of note, DNER  
425 involved in Notch pathway [45] was the only mediator tested in our study with a significantly  
426 decreased level in the lesions of the CL patients. The down-regulation of DNER may result in



the inhibition of Notch signaling pathway, which may contribute to the tissue damage observed in the lesions of CL patients.

In the case of chronic patients, seven proteins including CCL2, CXCL9, CXCL10, IL-18, CD40, TNFRSF9 and CST5 (cystatin D) were shown to have significant increasing rate in the lesions comparing with their normal skin counterparts.

By comparing the profile of inflammatory mediators in acute and chronic lesions, IL-8, IL-22RA1, PD-L1, TNF and GDNF showed significantly increased levels in the acute lesions. On the other hand, MCP-3, TNFRSF9 and TWEAK showed significantly decreased levels. .

Up-regulation of MCP-3/CCL7 was reported in the diffuse form of CL, and was suggested to be involved in the recruitment of immune cells during different phases of the disease. IL-8, IL-22RA1, PD-L1, TNF and GDNF were found to have significantly higher levels in the acute CL lesions compared to those of the chronic. MCP-3, TNFRSF9 and TWEAK were found to have significantly lower levels in the acute lesions. IL-22RA1 has been shown to play an important role in wound healing [46]. PDL1 was shown to regulate immune response against leishmaniasis caused by *L. mexicana* [47].

We observed an increase in the expression of OSM, TNFRSF9, OPG, CDCP1, MMP-10, ST1A1, HGF, CST5, TWEAK, GDNF and IL-22RA1 in CL. We also demonstrated the potential of our approach in detection of *Leishmania* specific IgG antibodies in the lesions of CL patients.

## 6. Conclusion

Herein, we report the development of a non-invasive sampling method combined with a high-throughput protein detection assay for profiling proteins involved in the inflammatory response in *L. tropica* caused lesions. Using this approach, we could profile inflammatory proteins in the

lesions from both active and chronic CL patient. Our data demonstrate that this non-invasive sampling method could be useful for identifying biomarkers for CL, albeit this approach would need to be evaluated and validated in a larger study in which control groups are included for other inflammatory/ulcerative skin lesions.

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## **\*Declaration of Interest Statement**

We confirm that the manuscript was approved by all authors and the authors declare no conflict of interest, and that the results presented in this manuscript have neither been published nor under consideration for publication elsewhere.

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